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Regulation of Cellular Oxidative Stress and Apoptosis by G Protein-Coupled Receptor Kinase-2; The Role of NADPH Oxidase 4

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Abstract

Cardiac myocyte oxidative stress and apoptosis are considered important mechanisms for the development of heart failure (HF). Chronic HF is characterized by increased circulating catecholamines to augment cardiac output. Long-term stimulation of myocardial β-adrenergic receptors (β-ARs) is deleterious in cardiac myocytes, however, the potential mechanisms underlying increased cell death are unclear. We hypothesize that GRK2, a critical regulator of myocardial β-AR signaling, plays an important role in mediating cellular oxidative stress and apoptotic cell death in response to β-agonist stimulation. Stimulation of H9c2 cells with a non-selective β-agonist, isoproterenol (Iso) caused increased oxidative stress and apoptosis. There was also increased Nox4 expression, but no change in Nox2, the primary NADPH isoforms and major sources of ROS generation in cardiac myocytes. Adenoviral-mediated overexpression of GRK2 led to similar increases in ROS production and apoptosis as seen with Iso stimulation. These increases in oxidative stress were abolished by pre-treatment with non-specific Nox inhibitor, apocynin, or siRNA knockdown of Nox4. Adenoviral-mediated expression of a GRK2 inhibitor prevented ROS production and apoptosis in response to Iso stimulation. β-arrestins are signaling proteins that function downstream of GRK2 in β-AR uncoupling. Adenoviral-mediated overexpression of β-arrestins increased ROS production and Nox4 expression. Chronic β-agonist stimulation in mice increased Nox4 expression and apoptosis compared to PBS or AngII treatment. These data demonstrate that GRK2 may play an important role in regulating oxidative stress and apoptosis in cardiac myocytes and provides an additional novel mechanism for the beneficial effects of cardiac-targeted GRK2 inhibition to prevent the development of HF.

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Disclosures

None of the authors has any conflicts of interest to disclose relevant to this work.

Keywords

G protein-coupled receptor kinases; oxidative stress; NADPH oxidase; heart failure

1. Introduction

Chronic heart failure (HF) is characterized by increased sympathetic nervous system activity with elevated levels of circulating catecholamines including norepinephrine and epinephrine [1]. Sustained β-adrenergic receptor (β-AR) stimulation in an attempt to improve ventricular function, subsequently leads to β-AR desensitization and downregulation which are hallmarks of end-stage HF [2]. Impaired β-AR signaling, in this setting, is mediated primarily by increased activity (2-3-fold) of G protein-coupled receptor kinase-2 (GRK2) which phosphorylates agonist-occupied receptors and targets them for uncoupling and degradation via β-arrestins [3,4]. GRK2 is known to be a critical regulator of cardiac function and inhibition of this kinase in transgenic mouse models and by cardiac-specific adenoviral-mediated gene delivery has been shown to rescue models of HF [5,6]. GRK2, also known as β-AR kinase-1 (βARK1), is a member of the family of serine-threonine kinases known as G protein-coupled receptor (GPCR) kinases which regulate GPCR signaling. Expression of the carboxyl-terminal 194 amino acids of βARK1, known as βARKct or GRK2ct, inhibits translocation of GRK2 to the membrane and restores signaling through β-ARs leading to enhanced myocardial function [7]. β-arrestins are scaffold signaling molecules that also play a key role in G protein-coupled receptor (GPCR) desensitization and downregulation downstream of GRK2. Phosphorylation of agonist-occupied β-ARs by GRK2 causes recruitment of β-arrestins which bind β-ARs, sterically inhibiting the receptor-G protein interaction and targeting the receptor for internalization [8]. β-arrestin expression has been shown to be significantly upregulated in an ischemic model of HF in rats [9].

It is well known that chronic β-agonist stimulation leads to cardiac myocyte apoptosis *in vitro* and *in vivo* [10,11] and apoptosis is thought to play an important role in the development of HF [12]. This has been shown to be specific for β_1-ARs whereas signaling through β_2-ARs has been shown to be cardioprotective [13]. Relatively little is known about the signaling pathways by which β-ARs regulate apoptosis in cardiac myocytes. Activation of adenylyl cyclase and protein kinase A (PKA) leading to intercellular Ca^{2+} overload is one proposed mechanism [14]. In addition, work in adult rat cardiac myocytes suggests that both mitochondria and reactive oxygen species (ROS) are involved in β-AR stimulated apoptosis [15]. Oxidative stress plays an important role in cardiac myocyte function and death and NADPH oxidases are the major source of O_2^- production [16]. NADPH oxidase (Nox) 2 and 4 are expressed in the heart [17] and upregulation of Nox4 by hypertrophic stimuli has been shown to promote apoptosis and mitochondrial dysfunction in cardiac myocytes [18]. Nox4 has also been shown to be a major source of oxidative stress in the failing heart and is expressed primarily in the mitochondria [19].

This study investigates the potential role of Nox-induced oxidative stress in β-agonist stimulated cardiac myocyte apoptosis with a particular focus on the regulation of cellular

oxidative stress and subsequent apoptosis by GRK2 as this kinase is upregulated in HF and plays a key role in regulating myocardial β-AR signaling and function.

2. Materials and Methods

2.1. Ethic statement

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

2.2. Reagents

All Cell culture reagents were purchase from Invitrogen Technologies (Eugene, OR) except Fetal Bovine Serum (FBS), which was obtained from Atlanta Biologicals (Lawrenceville, GA). Unless stated otherwise, all additional chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All antibodies and lentivirus particles were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Embryonic rat heart derived H9c2 cells were purchased from American Type Culture Collection (Manassas, VA).

2.3. Cell Culture

A stock of embryonic rat heart derived H9c2 cells was cultured in Dulbecco's Modified Eagle's Medium (DMEM) that was supplemented with 10% FBS, antibiotics (50U/ml penicillin and 50μg/ml streptomycin), and 2mM L-Glutamine at 37°C in a regular tissue culture incubator with an atmosphere of 95% air and 5% CO_2. Stock was stored in two 75 cm^2 flasks, and medium was replaced every 2-3 days. Prior to experiments, cells were serum starved 6-12 hours. All experiments were done in 2.5% FBS.

2.4. Drug Treatment Protocol

H9c2 cells stock was split onto 60mm dishes and grown to desired confluence in 5ml of supplemented DMEM. Cells were treated with Isoproterenol (Iso) to reach a final concentration of 10μM. Cells were collected following 1, 3, 6, 12, 24, 48, and 72 hours. For treatments longer than 24 hours cells were restimulated with Iso once every 24 hours until collection. Two control plates that did not receive isoproterenol treatment were harvested along with the 24 hour treatment plates. All subsequent experiments were done with either 24 hour treatments of either no drug, 10 μM isoproterenol, 1 μM angiotensin II, 20 μM forsoklin.

2.5. Adenoviral Infections of Cell Cultures

For adenoviral over-expression or inhibition of GRK2, cells were infected at a MOI of 10 with either wild type GRK2 (Ad-GRK2), inhibitor of GRK2 activity (Ad-GRK2ct), or empty (Ad-Null) adenoviruses for 24 hours prior to stimulation with 10 μM isoproterenol. For adenoviral over-expression of β-arrestin1 and β-arrestin2, cells were infected at a MOI of 10 with either wild type β-arrestin1 (Ad-βarr1), wild type β-arrestin2 (Ad-βarr2), or empty (Ad-Null) adenoviruses for 24 hours prior to stimulation with 10 μM isoproterenol.

2.6. Lentiviral Infections of Cell Cultures

For lentiviral inhibition of Nox4, cells were infected at a MOI of 10 with either a non-targeting scrambled sequence (Scr) or with a shRNA specific for Nox4 (siNox4) containing lentivirus for 18-24 hours before stimulation with isoproterenol or co-infection with adenovirus.

2.7. siRNA transfection

Predesigned target-specific siRNA duplexes of rat GRK2 (Cat#4390771) was obtained from Ambion to knock-down GRK2 protein expression. Scrambled oligo-ribonucleotide complex was also obtained from Ambion (Cat#4390843), which was not homologous to any mammalian genes and utilized as control. Cells were transfected with Lipofectamine 2000 (Invitrogen; CA, USA), according to manufacturers' instructions. Silencing was quantified by immunoblotting. Only experiments with verified silencing were used.

2.8. Osmotic Pumps

For the in vivo studies, 6 week old C57BL-6N males (Charles River, Wilmington, MA) were used. The mice were anesthetized and osmotic pumps (DURECT, Cupertino, CA) containing 100 μl of either PBS, angiotensin II (AngII) or isoproterenol dissolved in 0.4 mmol/L ascorbic acid were implanted subcutaneously. AngII was administered at a dose of 1.8 mg/kg/day. Isoproterenol was administered at a dose of 17 mg/kg/day. After ten days of drug administration, the mice were sacrificed and the hearts were excised, weighed and frozen in liquid nitrogen or sent for paraffin embedding.

2.9. Beta-Blockers and Apocynin

Cells were grown on 0.3% gelatin treated glass bottom dishes (MatTek, Ashland, MA). Cells were serum starved for 24 hours. After serum starvation, cells were treated with 100 μM Apocynin, 10 μM Metoprolol, or 10 μM ICI-118551, a β_2-AR selective antagonist, for 30 minutes. After treatment, cells were stimulated with 100 μM Isoproterenol for 10 minutes. Oxidative stress was then measured using MitoSOX (Invitrogen).

2.10. Protein Immunoblotting

Cells were lysed in buffer containing 25 mM HEPES, 1 mM EDTA, 125 mM NaCl, 0.5 mM NaF, 0.25% Nonidet P-40, 5% glycerol (pH 6.8), 10 μg/ml leupeptin, 20 μg/ml aprotinin and 1mM phenyl-methylsulfonyl fluoride. Equal amounts of protein for each sample were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted. Bands were visualized with ECL Western blotting substrate (Thermo Scientific, Rockford, IL). Band intensity was quantitated using NIH ImageJ software. GAPDH or α-tubulin was used as a loading control.

2.11. Immunoprecipitation

Cells were lysed in buffer containing 25 mM HEPES, 1 mM EDTA, 125 mM NaCl, 0.5 mM NaF, 0.25% Nonidet P-40, 5% glycerol (pH 6.8), 10 μg/ml leupeptin, 20 μg/ml aprotinin and 1mM phenyl-methylsulfonyl fluoride. Lysates were centrifuged at 15,000×g for 10 minutes, and supernatants were collected. The supernatant (approximately ~1 mg of protein) was

precleared with Protein A/G PLUS-Agarose (Santa Cruz) and then Nox4 mAb was added to the lysate for 4°C overnight with agitation. Each sample was centrifuged at 5000×g for 10 minutes to remove precipitated protein during overnight incubation. 20 μl pre-washed Protein A/G PLUS-Agarose was added to all samples, followed by overnight agitation at 4°C. Samples were then pelleted and washed with co-IP lysis buffer for three times. Samples were resuspended and heated in Laemmli buffer and subjected to Western blot analysis.

2.12. Caspase-3 Activity

Caspase-3 activity was assessed by a fluorometric assay from EMD Millipore (Billerica, MA). Cells were lysed in buffer containing the same concentrations used for protein immunoblotting. 50 μL of lysed cells were added to 96 well plates. 50 μL of assay buffer was then added to the plate. Cells were then incubated with Caspase-3 Fluorescent Substrate Conjugate at 37°C for 1 hour. Fluorescent reading was taken at excitation/emission maxima of 405/505 nm on a Tecan Safire 2. Readings were then normalized to protein concentration.

2.13. Anti-8-oxo-dg Staining

Cells were plated onto cover slips in a 12-well plate. Cells were serum starved for 6 hours. After 24 hours of 10μM isoproterenol treatment, cells were fixed with 1:1 Methanol:Acetone at −20°C. Fixed cells were treated with 0.05N HCl and subsequently washed numerous times with PBS. Cells were treated with 100μg/ml RNAse in 150mM NaCl, 15mM sodium citrate for 1 hour at 37°C. Cells were washed with PBS, 35% EtOH, 50% EtOH, and 75% EtOH. DNA was denatured with 0.15N NaOH in 70% EtOH. Cells were then treated with 70% (containing 4% v/v formaldehyde), 50% and 35% EtOH. Cover slips were treated with 5μg/ml proteinase K in 20mM Tris, 1mM EDTA, pH 7.5 (TE). Cells were then washed several times with 1XPBS and blocked with 5% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, cells were incubated with anti-8-hydroxyguanine antibody (1:250) in 1XPBS with 1% BSA, 0.01% Tween 20 at 4°C over night. Cover slips were washed with 1XPBS + 0.05% Tween-20. Cells were incubated with 5 μg/ml goat anti-mouse IgG Alexa Fluor 488 (Invitrogen, Eugene, OR) in 1XPBS + 1% BSA for 1 hour at room temperature. Cells were washed and mounted with Fluoroshield and imaged using an Olympus DSU Spinning Disk Confocal. The average fluorescence intensity of 8-oxo-dg staining per field for each experimental group of cells was calculated using NIH ImageJ analysis software and normalized against the total number of cells in each field. At least 4–5 random fields/condition were chosen for each experiment. The results are represented as fold change in fluorescence intensity from control.

2.14. MitoSOX Staining

Cells were grown on 0.3% gelatin treated glass bottom dishes. After isoproterenol treatment, cells were washed with HBSS. Cells were loaded with 5 μM MitoSOX Red (Invitrogen) and 1:1000 dilution of Hoechst 33342 (Invitrogen) in HBSS for 10 min at 37°C protected from the light. The cells were washed gently three times with warm buffer and placed in warm buffer for imaging. The indicator was detected using a confocal microscope at and excitation/emission maxima of 510/580 nm. The average fluorescence intensity of MitosSOX staining per field for each experimental group of cells was calculated using NIH ImageJ analysis software and normalized against the total number of cells in each field. At

least 4–5 random fields/condition were chosen for each experiment. The results are represented as arbitrary fluorescence units per total cell number.

2.15. CellROX Deep Red

Cells were grown on 0.3% gelatin treated glass bottom dishes. After isoproterenol treatment, cells were incubated with 2.5 μM CellROX Deep Red and 1:1000 dilution of Hoechst 33342 for 20 minutes at 37°C protected from the light. Dishes were then washed twice with PBS before fixing with 3.7% paraformaldehyde. After several washes in PBS, cells were imaged in warm buffer using a confocal microscope at the excitation/emission maxima of 644/664 nm. The average fluorescence intensity of MitosSOX staining per field for each experimental group of cells was calculated using NIH ImageJ analysis software and normalized against the total number of cells in each field. At least 4–5 random fields/ condition were chosen for each experiment. The results are represented as arbitrary fluorescence units per total cell number.

2.16. Immunostaining and Confocal Microscopy

Cells were washed, fixed in 3.7% paraformaldehyde and quenched. Cells were washed and then blocked for 2 hours in PBS with 10% FBS and 0.3% Triton X-100. After several washes with PBS + 0.3% Triton X-100, cells were treated with the primary antibody (Nox4 1:50 dilution, β-arrestin1 1:200 dilution, β-arrestin2 1:200 dilution) overnight at 4°C. Thereafter with either Alexa-Fluor 594 goat anti-rabbit (1:500), goat anti-rabbit IgG-FITC (1:100), or donkey anti-goat (1:500) secondary antibody for 1 hour. After three washes with PBS +0.3% Triton X-100, cells were mounted in Fluoroshield with DAPI. Cells were visualized using an Olympus DSU Spinning Disk Confocal.

2.17. RNA Extraction and Real-time PCR analysis

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA (1 μg) was used for the first-strand cDNA synthesis (Applied Biosystems, Carlsbad, CA, USA). Quantitative RT-PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Each cDNA template was amplified in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) with Nox4 primers. GAPDH was used to normalize the value. The PCR primer sequences used are as follows: for rat Nox4 5′- TTCTCAGGTGTGCATGTAGC -3 (forward), 5′- CGGAACAGTTGTGAAGAGAAGC -3 (reverse) and GAPDH 5′- CTGCACCACCAACTGCTTAC -3′ (forward) and 5′- CAGAGGTGCCATCCAGAGTT -3′ (reverse).

2.18. GRK2 Activity by Rhodopsin Phosphorylation

H9c2 cells were lysed in buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM phenyl-methylsulfonyl fluoride. Cell lysates (60μg of protein) were incubated with rhodopsin-enriched rod outer-segment membranes in a reaction buffer containing the following (in mmol/l): 10 MgCl2, 20 Tris•HCl, 2 EDTA, 5 EGTA, 0.1 ATP, and [−32P] ATP. After an incubation period of 30 mins in white light at room temperature, reactions were quenched

with ice-cold lysis buffer and centrifuged for 15 min at 13,000 g. Sedimented proteins were resuspended in 20 μl of protein gel-loading dye and subjected to 12% SDS-PAGE. Phosphorylated rhodopsin was visualized by autoradiography of dried polyacrylamide gels. Rod outer segments were obtained from Invision BioResources (Seattle, WA, USA). Each kinase activity reaction mixture contained 50 μg of purified rhodopsin.

2.19. Statistical Analysis

All data are expressed as mean ± SEM and were analyzed using Student's *t* test. Values of $p<0.05$ were considered significant.

3. Results

3.1. Upregulation of cellular oxidative stress by β-agonist stimulation in cardiac myocytes

It is well described that GRK2 regulates β-AR signaling by phosphorylating agonist-occupied receptors and targeting them for desensitization or uncoupling by β-arrestins [3,4]. Stimulation of H9c2 cardiac myocytes by the non-selective β-agonist, isoproterenol (10μM), led to an increase in GRK2 activity over a time course from 1 to 24 hours as measured by rhodopsin phosphorylation (Figure 1A). All subsequent β-agonist stimulation studies were done at the 24 hour time point. Using the CellROX assay, total cellular oxidative stress was increased 2.5-fold following isoproterenol treatment (Figure 1B). To investigate the potential mechanism of this oxidative stress, cells were stained with MitoSOX to quantitate a mitochondrial source of reactive oxygen species (ROS) generation. Indeed, mitochondrial superoxide levels remained significantly increased up to 72 hours with isoproterenol stimulation (Figure 1C). Staining for 8-hydroxygaunine (8-oxo-dg) showed a nearly 2-fold increase in oxidative DNA damage induced cell death by 24 hours of β-agonist stimulation (Figure 1D) and there was a significant upregulation in caspase-3 activity (Figure 1E), indicative of increased apoptotic cell death.

3.2. Nox4 is a primary source of β-agonist-induced reactive oxygen species

Stimulation of H9c2 cardiac myocytes with isoproterenol (10μM) from 1 to 24 hours led to a sustained increase in Nox4 expression which peaked at approximately 2-fold by 12 hours relative to untreated control cells (Figure 2A). Since Nox4 is constitutively active, this increase in expression appears to be an important mechanism of β-agonist-mediated oxidative stress. In addition to protein immunoblotting of cell lysates, immunostaining was done for Nox4 expression and was markedly increased by isoproterenol treatment (Figure 2B). To determine if this increase in Nox4 expression is specific to β-AR/Gαs stimulation, cardiac myocytes were stimulated with 1 μM of the Gq-coupled receptor agonist angiotensin II (Ang II) for 24 hours. As shown in Figure 2C, Ang II stimulation did not lead to significant upregulation in Nox4 expression and neither isoproterenol nor Ang II treatment altered Nox2 expression, the other major Nox isoform in the heart. Both Ang II and isoproterenol treatment significantly increased mitochondrial superoxide generation (Figure 2D). Cells were also stimulated with forskolin (20 μM) for 24 hours which directly activates adenylyl cyclase in order to investigate whether the β-agonist-stimulated increase in oxidative stress is mediated by cAMP/protein kinase A. This experiment demonstrated that forskolin did not increase mitochondrial ROS production (Figure 2D) as compared to the

significant increase induced by β-agonist stimulation. Similar findings were present for oxidative DNA damage-induced cell death (Figure 2E) and caspase-3 activity (Figure 2F). These data demonstrate that increased intracellular cAMP is not required for β-agonist-mediated mitochondrial ROS production and subsequent cell death.

3.3. β-adrenergic receptor subtype selectivity for mitochondrial ROS generation

We studied β-AR specificity for β-agonist-stimulated oxidative stress by pre-treating H9c2 cells with either the β_1-AR selective antagonist, metoprolol, or the β_2-AR selective antagonist ICI-118551 followed by stimulation with isoproterenol (10μM) for 24 hours. MitoSOX intensity was significantly diminished to control (untreated) levels following isoproterenol stimulation by pre-treatment with metoprolol (Figure 3A&B). In contrast, β_2-AR antagonist pre-treatment did not have any effect on mitochondrial ROS generation following β-agonist stimulation (Figure 3A&B). These data provide additional mechanistic evidence for the deleterious effects of chronic β_1-AR signaling in the heart and specifically with regard to increasing cellular oxidative stress and apoptotic cell death which play a role in the development of heart failure. β-blockade may also decrease ROS generation in cardiac myocytes and improve cell survival as a mechanism of the beneficial effects of β-blockers in cardiovascular disease.

3.4. Nox4 is a primary source of β-agonist-stimulated ROS production in cardiac myocytes

To more specifically investigate the role of Nox in β-adrenergic mediated oxidative stress and cell death, H9c2 cells were treated with the non-specific Nox inhibitor, apocynin, prior to stimulation by isoproterenol (10μM) for 24 hours. Apocynin inhibited essentially all of the increase in mitochondrial superoxide generation by β-agonist stimulation as measured by MitoSOX intensity (Figure 4A). There was no change in Nox4 expression levels with Apocynin treatment (Figure 4B). Based upon the previous finding of increased Nox4 expression in H9c2 cells following β-agonist stimulation (Figure 2A), we used an siRNA approach to specifically knockdown Nox4 expression. This led to a greater than 75% decrease in Nox4 protein expression by immunoblotting and immunostaining (Figure 4C). Using this genetic approach, the increase in Nox4 expression stimulated by isoproterenol was inhibited (Figure 4D) and this led to a significant inhibition in β-agonist-stimulated mitochondrial ROS generation as measured by MitoSOX intensity (Figure 4E). Mitochondrial ROS generation following isoproterenol stimulation of H9c2 cells after Nox4 knockdown was not different than unstimulated cells treated with scrambled siRNA as control, providing evidence that Nox4 may be the primary source of β-agonist-stimulated mitochondrial superoxide production. Knockdown of Nox4 also led to a significant inhibition of isoproterenol-induced oxidative DNA damage and cell death (Figure 4F) and caspase-3 activity (Figure 4G) which were not different from unstimulated control cells that were treated with scrambled siRNA.

3.5. Upregulation of GRK2 activity increases cellular oxidative stress and apoptotic cell death

We investigated whether GRK2 may play a role in regulating cellular ROS production following β-agonist stimulation as previous experiments demonstrated that increased intracellular cAMP/PKA activity did not lead to a significant change in oxidative stress.

Adenoviral-mediated overexpression of GRK2 in H9c2 cells similar to myocardial levels present in chronic heart failure (3-fold) led to a robust 2.5-fold increase in cellular oxidative stress measured by CellROX staining intensity compared to infection with the same number of total viral particles using a null adenovirus as control (Figure 5A). This increase in ROS generation by overexpression of GRK2 was similar to that of cells stimulated by isoproterenol for 24 hours (Figure 5A). Isoproterenol stimulation did not have any further effect on ROS generation in the setting of GRK2 overexpression. Similar increases in mitochondrial oxidative stress as measured by MitoSOX intensity (Figure 5B) as well as increases in cell death induced by oxidative DNA damage (Figure 5C) and caspase-3 activity (Figure 5D) were also seen with GRK2 overexpression. Importantly, inhibition of GRK2 activity by expression of the well-described inhibitor of GRK2, GRK2ct (also known as βARKct) [5,6], entirely inhibited the increase in cellular oxidative stress following β-agonist stimulation (Figure 5A). These results support the hypothesis that GRK2 may be an important regulator of myocardial oxidative stress under conditions of increased catecholamine stimulation as present in heart failure. The inhibition of GRK2 activity by GRK2ct also inhibited mitochondrial ROS generation in a similar fashion (Figure 5B). Expression of GRK2ct also inhibited cell death induced by oxidative DNA damage following β-agonist stimulation (Figure 5C) as well as caspase-3 activity (Figure 5D). We have shown that siRNA-mediated inhibition of GRK2 also inhibited caspase-3 activity following β-agonist stimulation (Figure 6A). We then investigated whether GRK2 plays a role in regulating Nox4 as a possible mechanism for GRK2-induced mitochondrial ROS generation. Interestingly, overexpression of GRK2 in H9c2 cells led to a 4-fold increase in Nox4 protein expression which was not different from the increase in Nox4 expression following isoproterenol stimulation (Figure 5E) and there was no additional increase in Nox4 expression following isoproterenol stimulation in the setting of GRK2 overexpression. Inhibition of GRK2 by expression of either GRK2ct or GRK2 siRNA inhibited the β-agonist-induced increase in Nox4 expression (Figure 5F, 6B, 6C) demonstrating that GRK2 may regulate cellular/mitochondrial ROS generation and subsequent oxidative DNA damage-induced cell death via Nox4. Isoproterenol stimulation or overexpression of GRK2 also led to a about 2-fold increase in Nox4 mRNA expression measured by RT-PCR (Figure 5G). Expression of GRK2ct inhibited the β-agonist-stimulated increase in Nox4 transcription. These data provide evidence that GRK2 plays a role in regulating the transcription of Nox4 following β-AR stimulation. Interestingly, we have also found that Nox4 interacts with GRK2 at the protein level under basal conditions and that this is increased following β-agonist (Iso) stimulation (Figure 6D). This data potentially suggests another mechanism of Nox4 regulation by GRK2 at the protein level.

3.6. Role of Nox4 in GRK2-mediated oxidative stress

In order to more specifically identify the primary mechanism for the regulation of cellular oxidative stress by GRK2, we performed experiments using simultaneous overexpression of GRK2 and siRNA-mediated knockdown of Nox4. As previously shown, isoproterenol stimulation of H9c2 cells led to a significant increase (> 2-fold) in mitochondrial superoxide generation as assessed by MitoSOX staining and this was similar to that seen by overexpression of GRK2 (Figure 7A). Knockdown of Nox4 inhibited any increase in ROS generation induced by overexpression of GRK2 and this was unchanged by β-agonist

stimulation (Figure 7A). These studies provide more mechanistic and direct evidence that mitochondrial oxidative stress induced by GRK2 is mediated by Nox4. Figures 7B and 7C show identical conditions of GRK2 overexpression and knockdown of Nox4 which leads to inhibition of the increase in oxidative DNA damage induced cell death and caspase-3 activity resulting from overexpression of GRK2. Figure 7D demonstrates the effectiveness of siRNA-mediated knockdown of Nox4 under conditions of null adenovirus (Ad-Null) or Ad-GRK2 infection. The increased expression of Nox4 previously seen with overexpression of GRK2 is prevented by knockdown of Nox4 using this siRNA approach.

3.7. In vivo effects of chronic β-agonist stimulation on Nox4 and cardiac myocyte apoptosis

To study the myocardial effects of chronic β-agonist stimulation on Nox4 as a potential mechanism for increased oxidative stress and apoptosis, we treated adult male wild-type C57bl6 mice with isoproterenol (17mg/kg/day) for 10 days via a mini-osmotic pump. Angiotensin II (1.8mg/kg/day) stimulation was also studied as another hypertrophic agonist and the control group of mice was treated with PBS for the same 10 day duration. Treatment with either isoproterenol and angiotensin II led to a significant degree of hypertrophy compared to PBS treated controls (Figure 8A). Isoproterenol treatment led to an increase (approximately 2-fold) in myocardial Nox4 expression compared to controls (Figure 8B). In contrast, there was no significant increase in Nox4 expression in the angiotensin II treated group versus control despite a similar degree of hypertrophy as in the isoproterenol treated group. Both isoproterenol and angiotensin II treatment led to increased caspase-3 activity (Figure 8C) and apoptosis as measured by TUNEL staining (Figure 8D). These data preliminarily suggest that upregulation of Nox4 expression and increased cellular oxidative stress may be important mechanisms of apoptotic cell death and myocardial dysfunction that are associated with chronic β-agonist stimulation in the myocardium.

3.8. Overexpression of β-arrestin increases cellular oxidative stress

We investigated whether β-arrestins may play a role in regulating β-agonist stimulated increases in oxidative stress as β-arrestin1 and 2 are known to regulate β-AR signaling downstream of GRK2. Approximately 5-fold overexpression of β-arrestin1 or 2 was achieved in H9c2 following infection with Ad-βarr1 or Ad-βarr2 (Figure 9A). Adenoviral-mediated overexpression of β-arrestin1 or 2 in H9c2 cells led to a robust 1.5-2-fold increase in total cellular oxidative stress measured by CellROX staining intensity compared to infection with the same number of total viral particles using a null adenovirus as control (Figure 9B). This increase in ROS generation by overexpression of either β-arrestin1 or 2 was similar to that of cells stimulated by isoproterenol for 24 hours (Figure 9B). Isoproterenol stimulation did not have any further effect on ROS generation in the setting of β-arrestin1 or 2 overexpression. Similar increases were seen in mitochondrial superoxide generation as measured by MitoSOX staining intensity with β-arrestin1 or β-arrestin2 overexpression (Figure 9C). We then investigated whether β-arrestins play a role in regulating Nox4 as a possible mechanism for β-arrestin-induced mitochondrial ROS generation as was demonstrated for GRK2-induced mitochondrial ROS generation. Overexpression of β-arrestin1 (7-fold) in H9c2 cells led to a 2.4-fold increase in Nox4 protein expression and a 6-fold increase in β-arrestin2 led to a 1.2-fold increase in Nox4

(Figure 9D). These data demonstrate that β-arrestin signaling can lead to increased oxidative stress similar to the degree produced following β-agonist stimulation and in the setting of increased GRK2 activity. Therefore, β-agonist-stimulated ROS production by Nox4 may primarily be regulated by β-arrestin downstream of GRK2.

3.9. Expression of GRK2, Nox4 and β-arrestin1 are increased in failing human heart

As Nox4 has been shown to be a major source of oxidative stress in the failing heart and GRK2 is upregulated in HF, we investigated the expression of Nox4, GRK2, and β-arrestin1 in left ventricles from advanced HF patients compared to non-failing controls to provide additional translational relevance. There was a 3-fold increase in Nox4, 1.7-fold increase in GRK2, and a 1.8-fold increase in β-arrestin1 protein expression in the failing hearts versus normal control (Figure 10).

4. Discussion

The major finding of this study is the novel role for GRK2 and β-arrestins in regulating cellular and mitochondrial oxidative stress under conditions of chronic β-agonist stimulation. Signaling through β_1-ARs in cardiac myocytes led to increased mitochondrial ROS generation via upregulation of Nox4. This did not appear to be mediated by cAMP/PKA but rather a result of increased GRK2 activity. Overexpression of GRK2 led to a significant increase in Nox4 expression, ROS production, and oxidative DNA damage-induced cell death, which was similar to that caused by isoproterenol stimulation. Upregulation of Nox4 expression following β-agonist stimulation of cardiac myocytes was inhibited by the GRK2 inhibitor, GRK2ct, and oxidative stress and cell death induced by overexpression of GRK2 was inhibited by siRNA-mediated knockdown of Nox4. The *in vivo* studies also demonstrate myocardial upregulation of Nox4 with chronic β-agonist stimulation leading to increased caspase-3 activity and apoptotic cell death. There appears to be a transcriptional link between GRK2/β-arrestin and Nox4 and these data provide another mechanism for the deleterious effects of increased GRK2 activity in the myocardium. In addition, there appears to be a protein-protein interaction between GRK2 and Nox4 which is increased by β-agonist stimulation potentially suggesting another level of regulation of Nox4 by GRK2. These data provide additional evidence for the potential therapeutic role of GRK2 inhibition for the prevention or reversal of HF.

Under pathological conditions, mitochondria are the major source of ROS, which are generated primarily through electron leakage from the electron transport chain [20]. ROS are also produced by O_2^--producing enzymes such as NADPH oxidases and xanthine oxidase. The role of Nox4 in the development of pathological myocardial hypertrophy and HF has become more defined by recent work utilizing novel mouse models with cardiac-specific overexpression of Nox4 as well as knockout of Nox4 [18, 19]. Myocardial expression of Nox4 was increased by transverse aortic constriction (TAC) in mice as well as with aging [19]. This study also clearly demonstrated that Nox4 is primarily localized in mitochondria in cardiac myocytes. Nox4 is ubiquitously expressed and distinct from other members of the Nox family, it is believed to be constitutively active and does not require cytosolic factors such as $p47^{phox}$, $p67^{phox}$, and the small molecule GTPase Rac for its activation. Therefore,

its expression level essentially determines the amount of O_2^- production in cells. Work in these transgenic mice also suggested that increased O_2^- production in the mitochondria caused by Nox4 upregulation induces oxidative mitochondrial damage, including oxidation of mitochondrial proteins, thereby leading to mitochondrial dysfunction and apoptotic cell death [19]. The generation of mice with cardiac-specific deletion of Nox4 has also significantly improved the understanding of the role of endogenous Nox4 in the heart [18]. A primary finding of this study was that Nox4 localized in mitochondria in cardiac myocytes is a major source of O_2^- production in the heart. In particular, upregulation of Nox4 is primarily responsible for the increased mitochondrial O_2^- production in response to pressure overload in the mouse heart. In addition, Nox4 was found to play a critical role in mediating mitochondrial dysfunction, apoptosis in cardiac myocytes, and eventual left ventricular dysfunction in response to pressure overload.

Mechanisms by which Nox4 expression and activity are regulated in cardiac myocytes are not well understood. We believe our study provides a novel mechanistic link between chronic β-adrenergic stimulation, known to be deleterious in the heart, and mitochondrial oxidative stress and subsequent cell death. It has been well described that myocardial sympathetic activity is increased in chronic heart failure [1] and that elevated levels of circulating catecholamines are associated with increased mortality [21]. Impaired myocardial β-AR signaling is a hallmark of chronic HF occurring in the setting of both pressure overload and chronic ischemia and is due to increased expression and activity of GRK2 which phosphorylates agonist-occupied β-ARs and targets them for internalization by β-arrestins. This leads to downregulation of total β-AR density by approximately 50% and impaired signaling through remaining receptors. Previous studies have demonstrated a critical role of GRK2 in regulating myocardial function [22] and inhibition of GRK2 by GRK2ct has been shown to improve left ventricular function and rescue small animal models of HF [5,6]. In addition, cardiac-specific expression of GRK2ct in a mouse model of ischemia-reperfusion injury showed decreased apoptosis and infarct size [23]. A potential mechanism for the *in vivo* therapeutic success of GRK2 inhibition in small animal models of HF and myocardial infarction may be decreasing mitochondrial oxidative stress and myocyte apoptosis in the heart as a result of inhibiting Nox4 expression and activity. In addition to Nox4 in cardiac myocytes, this Nox isoform is also expressed in cardiac fibroblasts and may also play an important role in mediating cardiac fibrosis, a precursor to HF, by stimulating myofibroblast differentiation [24].

A recent study from Chen et al. demonstrated that GRK2 can localize to mitochondria and that this is increased after ischemic or oxidative stress [Chen et al. Circ Res 2013]. The ROS-mediated mitochondrial localization of GRK2 was found to be dependent on binding to Hsp90 that served as a chaperone protein. This interaction was dependent on ERK-mediated phosphorylation of GRK2 at the Ser670 residue at the carboxyl-terminus of the peptide. Localization of GRK2 to the mitochondria was associated with increased apoptosis *in vitro* and *in vivo*. Importantly, GRK2ct-mediated cardioprotection in a transgenic model of cardiac-specific overexpression of this peptide was found to be mediated via inhibition of mitochondrial GRK2 localization in response to ERK phosphorylation. Our findings provide additional mechanistic data on how GRK2 may be mediating increases in mitochondrial and cellular oxidative stress leading to cell death via Nox4. This adds to the previous literature

regarding mechanisms of cardiac myocyte apoptosis following chronic β_1-adrenergic receptor stimulation including the cAMP-PKA pathway [25] and Ca^{2+}/calmodulin-dependent protein kinase II signaling [26].

A limitation of this study is the use of H9c2 cells versus adult cardiac myocytes. These cells are phenotypically distinct from cardiac myocytes, even though they share several properties. H9c2 cells have provided technical advantages for studying cellular mechanisms, and they have often been used to investigate the pathways implicated in cardiac cell death secondary to oxidative stress [27, 28, 29, 30]. Additional studies in adult cardiac myocytes will provide further confirmation of this novel role of GRK2 in regulating cellular oxidative stress in the myocardium.

In summary, our results suggest that upregulation of Nox4 is an important mechanism of cellular and mitochondrial oxidative stress leading to apoptotic cell death in cardiac myocytes following chronic β-agonist stimulation. This does not appear to be due to increased intracellular cAMP/PKA activity but rather to increased GRK2 activity. Overexpression of GRK2 in H9c2 cells led to upregulation of Nox4 transcription and protein expression and subsequent mitochondrial oxidative stress and apoptosis similar to that seen with β-agonist stimulation. In contrast, inhibition of GRK2 prevented β-agonist-induced upregulation of Nox4 and the resulting mitochondrial ROS production and cell death. Importantly, this appeared to be selective for β_1-AR signaling as mitochondrial oxidative stress was not increased by β-agonist stimulation in the presence of a β_1-AR antagonist. Chronic isoproterenol treatment in mice also led to increased myocardial Nox4 expression, caspase-3 activity, and apoptosis. The increase in Nox4 expression was not present following stimulation by the pro-hypertrophic agonist AngII *in vitro* or *in vivo*. These data provide additional mechanistic evidence for the deleterious effects of increased GRK2 activity in the heart and for the potential therapeutic role of inhibiting myocardial GRK2 activity as an approach for the prevention or treatment of HF.

Acknowledgments

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Highlights

- GRK2 Regulates cellular oxidative stress and apoptotic cell death
- Nox4 is a primary source of β-agonist-stimulated ROS production in cardiac myocytes
- Overexpression of β-arrestins increased ROS production and Nox4 expression
- β-agonist-stimulated ROS production by Nox4 is regulated by β-arrestin downstream of GRK2

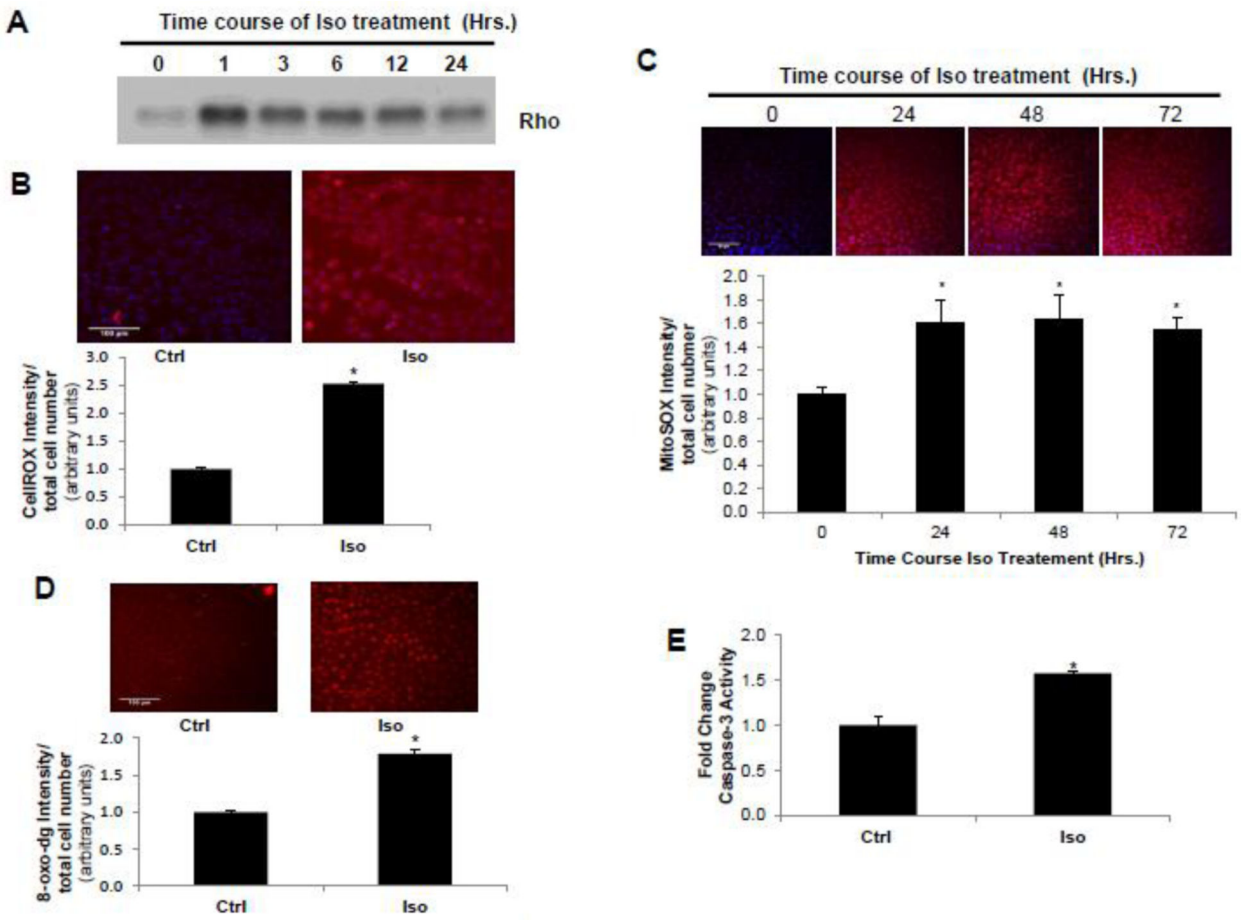


Figure 1. β-agonist stimulation upregulates GRK2 activity and oxidative stress in cardiac myocytes

A, Representative autoradiograph demonstrating GRK2 activity measured by rhodopsin (Rho) phosphorylation in H9c2 cells treated with 10μM β-AR agonist, isoproterenol (Iso). *B*, Confocal images of H9c2 cells stained with CellROX (*red*) showing increased total oxidative stress following Iso treatment (*upper panel*). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n=3; *p<0.00001 vs. Control (Ctrl). *C*, Confocal images of H9c2 cells stained with MitoSOX (*red*) showing increase in mitochondrial oxidative stress following Iso treatment for 24, 48, and 72 hours (*upper panel*). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n≥3; *p<0.04 vs. Control (0 Hrs.). *D*, Confocal images (*upper panel*) of 8-hydroxyguanine (8-oxo-dg) stained *red* with Alexa Fluor 594 dye are shown in Ctrl and Iso treated H9c2 cells. Fluorescence quantitation shown below. n=3; *p<0.0005 vs. Ctrl. *E*, Caspase-3 fluorometric assay shows increase in caspase-3 activity following Iso treatment. n= 3; *p=0.004 vs. Ctrl.

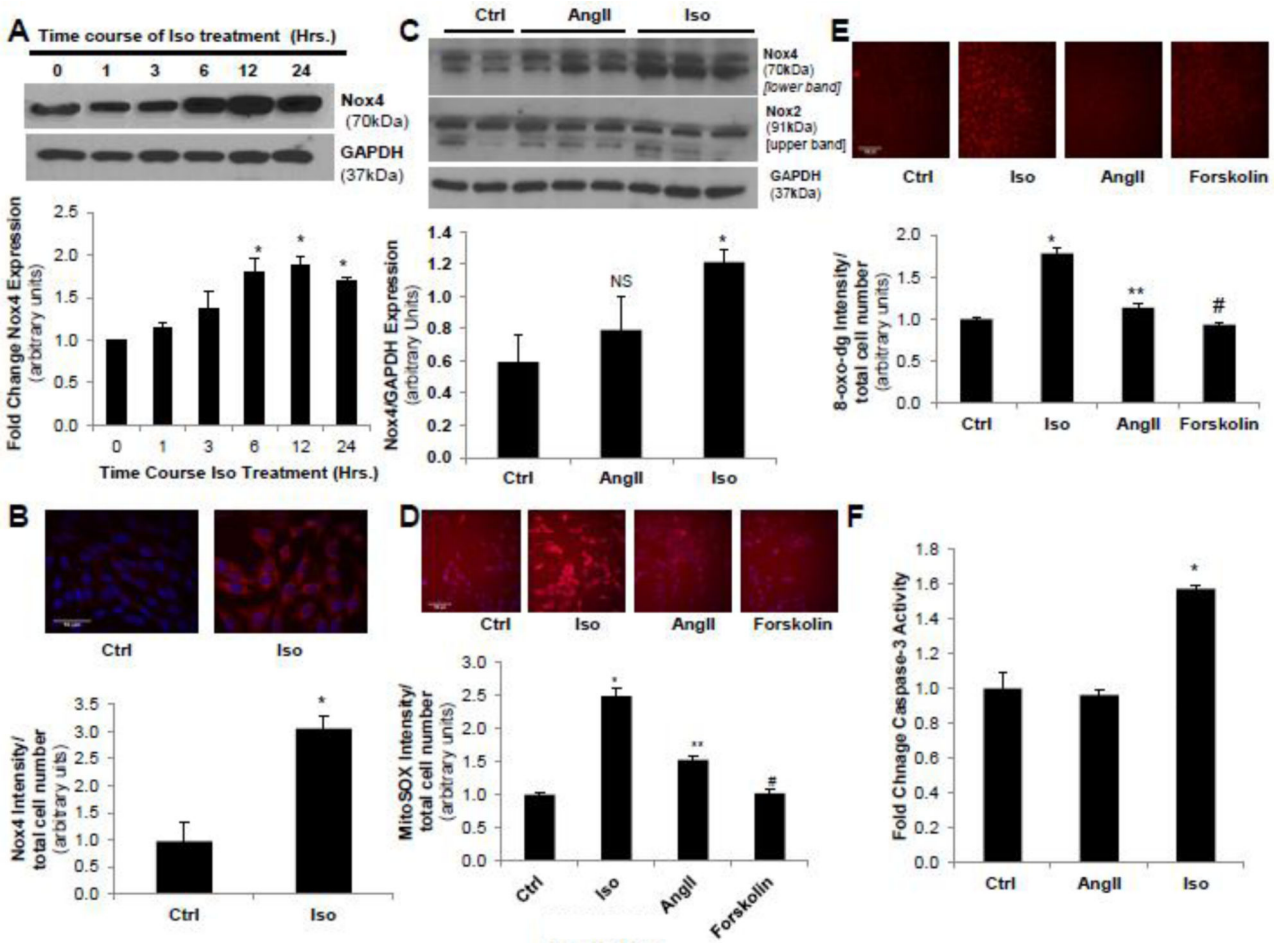


Figure 2. Nox4 is upregulated following β-agonist stimulation and oxidative stress is increased in a cAMP-independent manner

A, Representative immunoblot (*upper panel*) showing a time course of increasing Nox4 expression with β-AR agonist, isoproterenol (Iso) stimulation. GAPDH was used as a loading control. Densitometric analysis (*lower panel*) of Nox4 normalized to GAPDH. n=2 at each time point; *p<0.02 vs. Control (0 Hrs.) *B*, Confocal images (*upper panel*) of Nox4 stained with *red* Alexa Fluor 594 dye are show in Control (Ctrl) and Iso treated H9c2 cells. Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n=3; *p=0.007 vs. Ctrl. *C*, Representative immunoblot (*upper panel*) showing increased Nox4 but not Nox2 expression with Iso stimulation compared to untreated control (Ctrl). AngII did not lead to a significant change in Nox4 expression vs. Ctrl. GAPDH was used as a loading control. Densitometric analysis (*lower panel*) of Nox4 normalized to GAPDH. n=3; *p<0.01 vs. Ctrl; NS (p not significant vs. Ctrl). *D*, Confocal images of H9c2 cells stained with MitoSOX (*red*) showing increased mitochondrial oxidative stress following Iso and AngII treatment but not direct activation of adenylyl cyclase with Forskolin (*upper panel*) . Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n≥3; *p<0.0002 vs. Ctrl, **p=0.0011 vs. Ctrl, #p<0.0001 vs. Iso & p>0.05 vs. Ctrl. *E*, Confocal images (*upper panel*) of 8-hydroxyguanine (8-oxo-dg) stained *red* with Alexa Fluor 594 dye are show in Ctrl, Iso, AngII and Forskolin treated H9c2 cells. Fluorescence quantitation

shown below. n=3; *p<0.0007 vs. Ctrl, **p<0.05 vs. Ctrl, #p=0.0003 vs. Iso & p>0.05 vs. Ctrl. F, Caspase-3 fluorometric assay shows increase in caspase-3 activity following Iso but not AngII treatment. n=3; *p<0.005 vs. Ctrl and p<0.0001 vs. AngII.

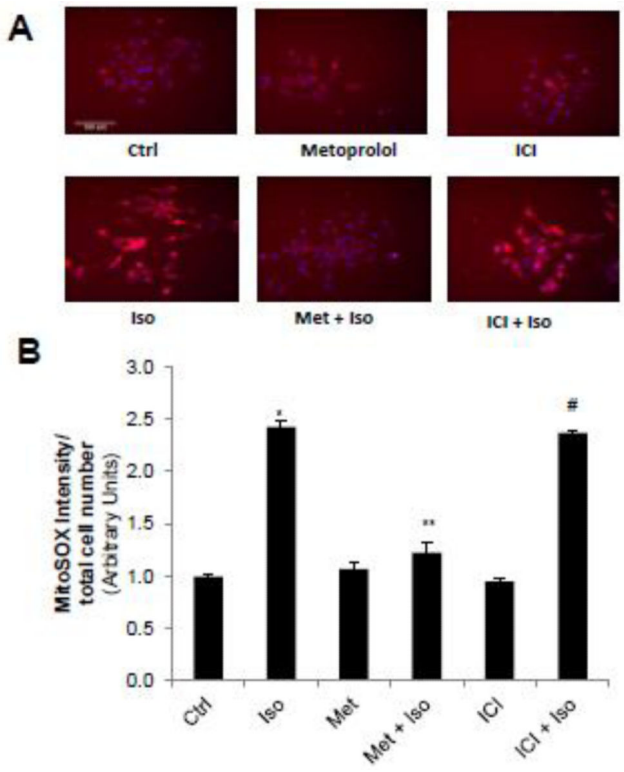
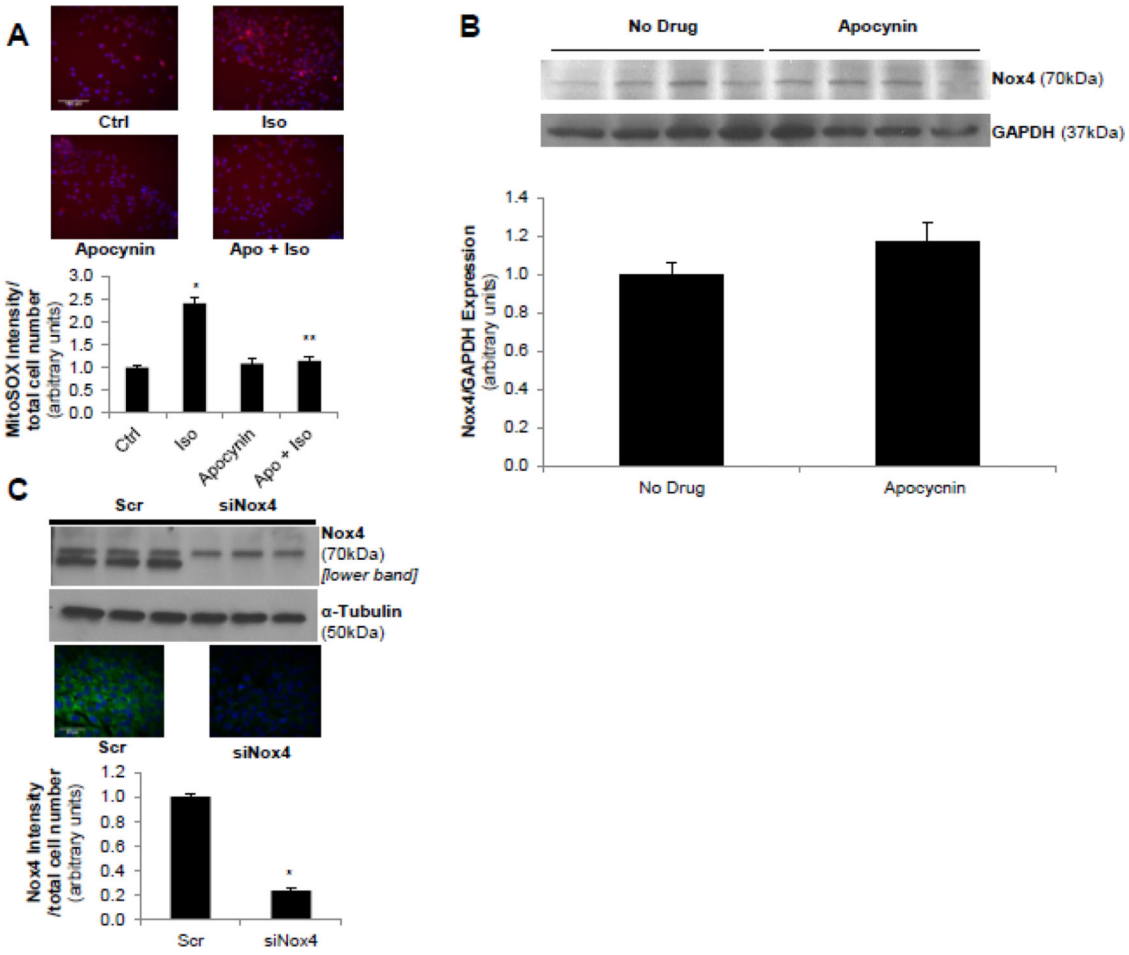


Figure 3. Increases in Mitochondrial Oxidative Stress are β_1-Adrenergic Receptor (β_1-AR)-Specific

A, Confocal images of H9c2 cells stained with MitoSOX (*red*) showing basal and β-AR agonist, isoproterenol (Iso) stimulated mitochondrial oxidative stress in Control (Ctrl), β_1-AR selective antagonist, Metoprolol (Met), and β_2-AR selective antagonist, ICI-118551 (ICI) treated cells. Nuclei are stained *blue* with Hoechst 33342. *B*, Fluorescence quantitation showing Iso stimulated increases in mitochondrial oxidative stress are inhibited by β_1-AR blockade but not β_2-AR blockade. n=3; *p<0.0001 vs. Ctrl, **p=0.0005 vs. Iso, #p<0.0001 vs. ICI.



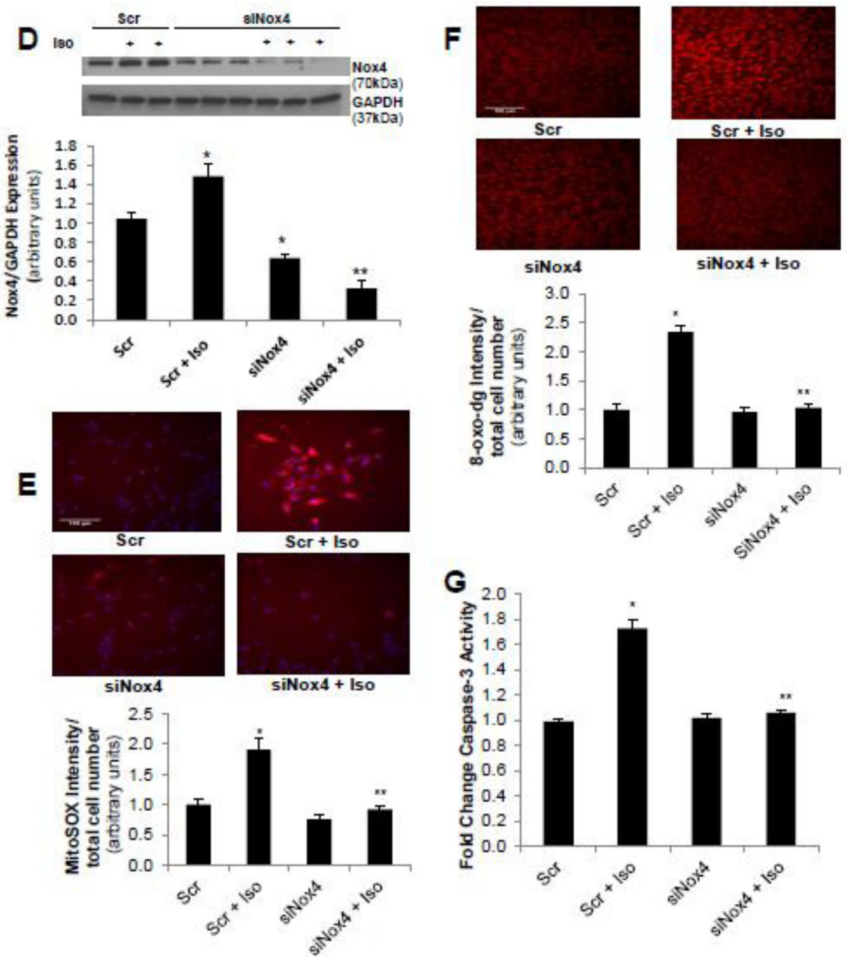


Figure 4. Nox4 is a primary source of β-agonist induced mitochondrial oxidative stress and apoptotic signaling

A, Confocal images of H9c2 cells stained with MitoSOX (*red*) showing basal and isoproterenol (Iso)-stimulated mitochondrial oxidative stress in Control (Ctrl) and Apocynin (Apo) treated cells. Apo is a non-selective Nox inhibitor. Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n=3; *p<0.001 vs. Ctrl, **p=0.002 vs. Iso. *B*, Representative immunoblot (*upper panel*) showing increased Nox4 expression following Apocynin treatmnent. GAPDH was used as a loading control. Densitometric analysis shown below. n=4; p=0.14 Apocynin vs. No Drug. *C*, Representative immunoblot (*upper panel*) showing knockdown of Nox4 expression with Nox4 siRNA (siNox4) vs. scrambled control (Scr). α-tubulin was used as a loading control. Confocal images (*middle panel*) of Nox4 stained with*green* with FITC are show in Scr and siNox4 transfected H9c2 cells. Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n=3 in each group, *p<0.0001 vs. Scr.*D*, Representative immunoblot (*upper panel*) showing inhibition of Iso stimulated upregulation of Nox4 expression following siNox4. GAPDH was used as a loading control. Densitometric analysis (*lower panel*) of Nox4 normalized to GAPDH. n=3; *p<0.02 vs. Scr, **p<0.001 vs. Scr + Iso. *E*, Confocal images H9c2 cells stained with MitoSOX (*red*) showing inhibition of Iso stimulated mitochondrial oxidative stress with Nox4 knockdown (siNox4) vs. scrambled control (Scr) (*upper panel*) . Nuclei are

stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n≥3; *p=0.01 vs. Scr, **p<0.007 vs. Scr + Iso. *F*, Confocal images (*upper panel*) of 8-hydroxyguanine (8-oxo-dg) stained *red* with Alexa Fluor 594 dye are shown in Iso treated H9c2 cells following transfection with siNox4 or Scr. Fluorescence quantitation shown below. n=3; *p<0.001 vs. Scr, **p<0.0007 vs. Scr + Iso. *G*, Caspase-3 fluorometric assay showing no increase in caspase-3 activity with Iso stimulation following siRNA Nox4 knockdown. n=3; *p<0.0004 vs. Scr, **p<0.0006 vs. Scr + Iso.

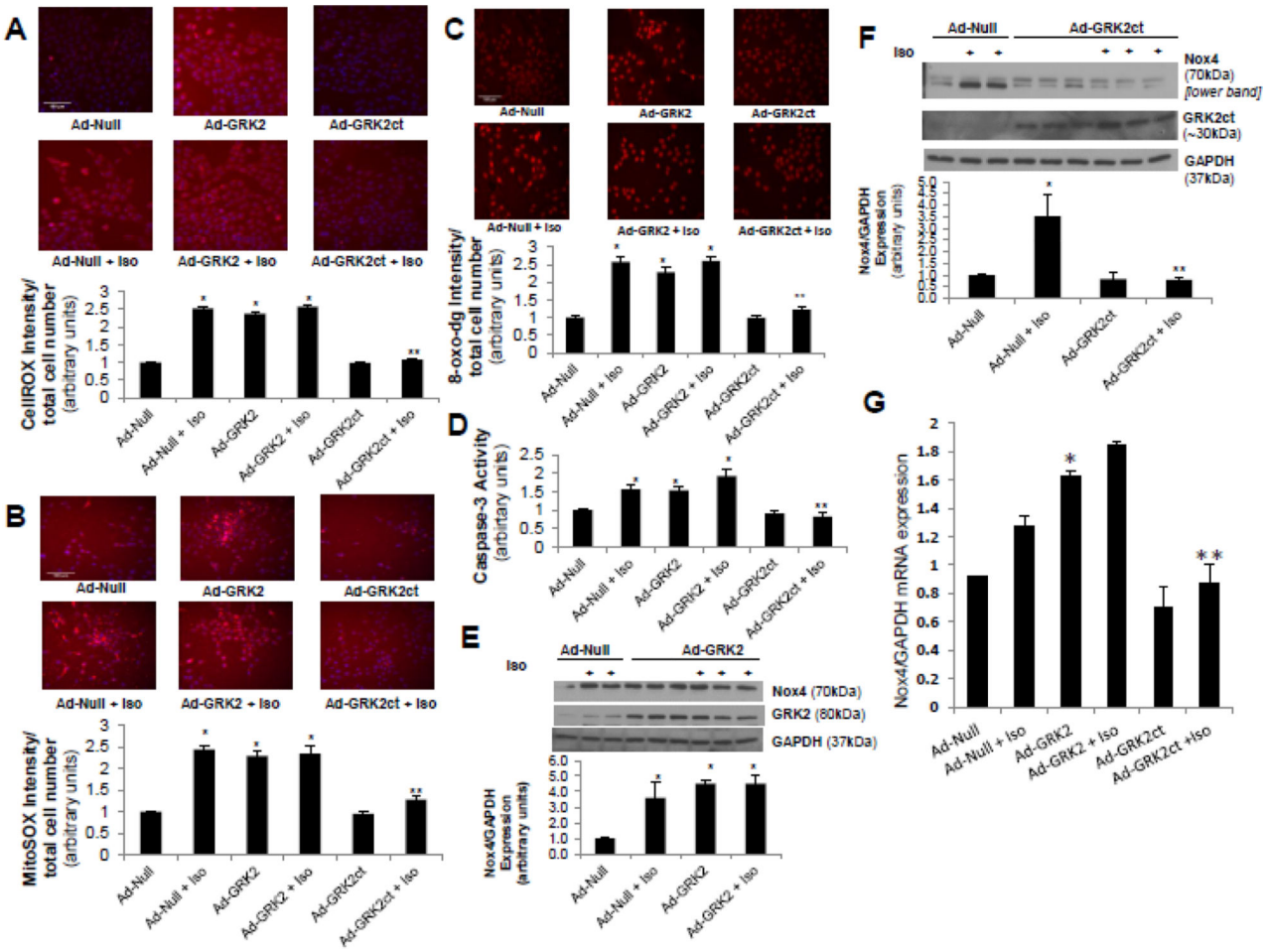


Figure 5. GRK2 regulates β-AR-agonist stimulated oxidative stress and apoptotic cell death

A, H9c2 cells infected with adenoviruses overexpressing GRK2 (Ad-GRK2), GRK2 inhibitor, GRK2ct, (Ad-GRK2ct), or null virus (Ad-Null). Confocal images of CellROX (*red*) showing basal and β-AR agonist, isoproterenol (Iso) stimulated total oxidative stress (*upper panel*). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n=3; *p<0.0001 vs. Ad-Null, **p<0.0001 vs. Ad-Null + Iso. *B*, Confocal images showing MitoSOX (*red*) staining in H9c2 cells transfected with Ad-GRK2, Ad-GRK2ct or Ad-Null viruses. Nuclei are stained *blue* with Hoechst 33342. Fluorescence shown below. n≥3; *p<0.01 vs. Ad-Null, **p<0.0002 vs. Ad-Null + Iso. C, Confocal images (*upper panel*) of 8-hydroxyguanine (8-oxo-dg) stained *red* with Alexa Fluor 594 dye are shown in Iso treated H9C2 cells following transfection with Ad-GRK2, Ad-GRK2ct, or Ad-Null. Fluorescence quantitation shown below. n=3; *p<0.002 vs. Ad-Null, **p<0.002 vs. Ad-Null + Iso. D, Caspase-3 fluorometric assay showing an increase in caspase-3 activity following GRK2 overexpression or Iso stimulation and inhibition of Iso effects with GKR2 inhibition. n≥3; *p<0.003 vs. Ad-Null, **p=0.002 vs. Ad-Null +Iso. *E*, Representative immunoblot (*upper panel*) showing increased Nox4 expression with Ad-GRK2 vs. Ad-Null. GAPDH was used as a loading control. Densitometric analysis (*lower panel*) of Nox4 normalized to GAPDH. n=3; *p<0.03 vs. Ad-Null F, Representative immunoblot (*upper panel*) showing inhibition of Iso stimulated Nox4 expression with Ad-GRK2ct vs. Ad-Null. GAPDH was

used as a loading control. Densitometic analysis (*lower panel*) of Nox4 normalized to GAPDH. n=3; *p<0.03 vs. Ad-Null, **p=0.02 vs. Ad-Null + Iso. *G*, Quantitative PCR showing Nox4 mRNA expression under basal conditions and with Iso stimulation with GRK2 or GRK2ct overexpression. Values normalized to GAPDH. *p=0.001 vs. Ad-Null, **p=0.009 vs. Ad-Null + Iso. n=3 for all groups.

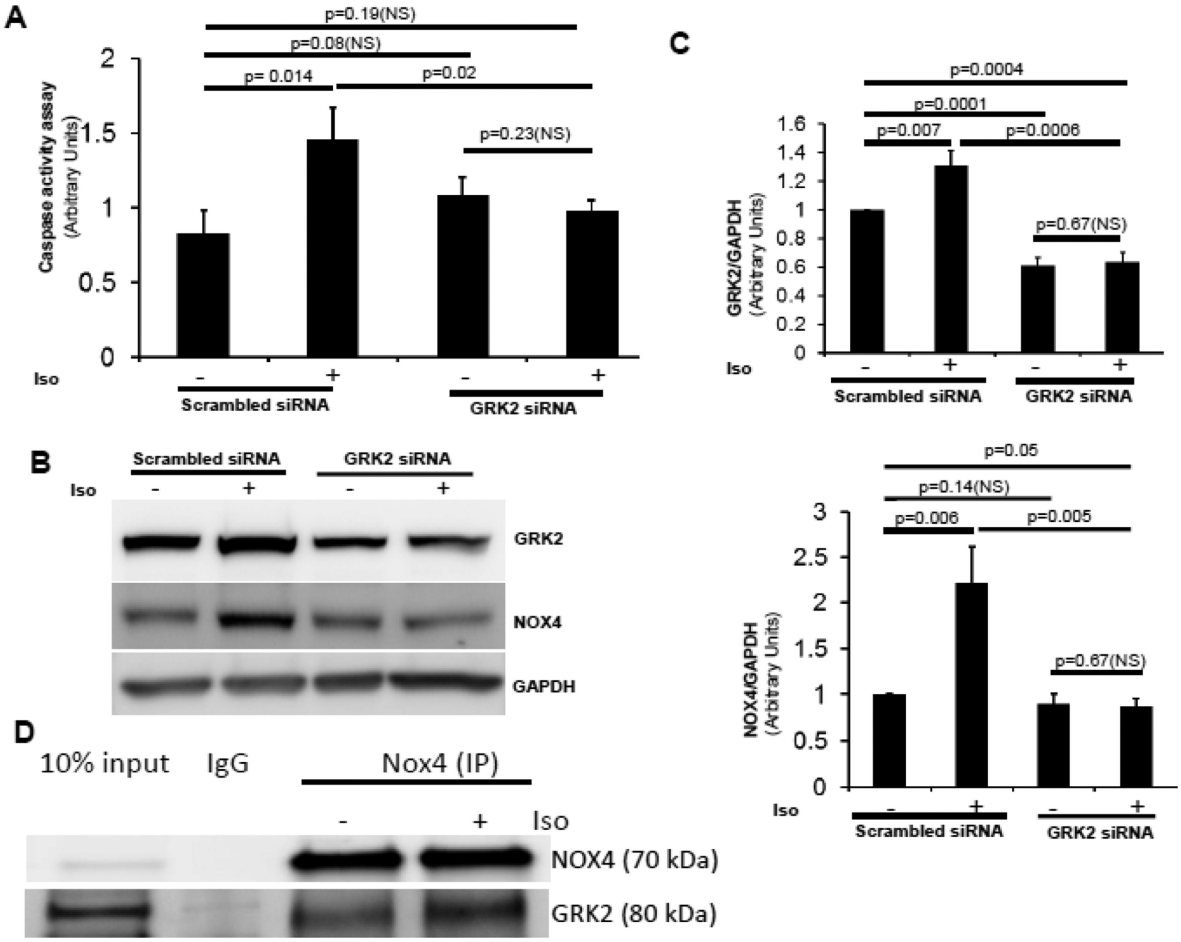


Figure 6. Knockdown of GRK2 inhibits caspase-3 activity and Nox4 expression following β-agonist stimulation

A, Caspase-3 fluorometric assay showing decrease in caspase-3 activity with GRK2 inhibition and Iso treatment. n≥3; p=0.014 vs. scrambled siRNA - Iso, p=0.02 vs. GRK2 siRNA + Iso. B, Representative immunoblot showing increased GRK2 expression with Iso treatment in scrambled siRNA but not in GRK2 siRNA. GAPDH was used as a loading control. Representative immunoblot showing inhibition of Iso stimulated Nox4 expression with GRK2 siRNA vs. scrambled siRNA. GAPDH was used as a loading control. C, Densitometic analysis of GRK2 normalized to GAPDH. n=3; p=0.007 vs. scrambled siRNA - Iso, p=0.0006 vs. GRK2 siRNA + Iso. Densitometric analysis of Nox4 normalized to GAPDH. n=3; p=0.006 vs. scrambled siRNA, p=0.005 vs. GRK2 siRNA + Iso. *D,* Lysates from Iso treated and untreated were incubated with either rabbit monoclonal antibodies directed against Nox4 (IP) or rabbit IgG (Control), and antibodies were precipitated with pre-washed Protein A/G PLUS-Agarose as described under “Materials and Methods.” Immunoprecipitates were separated by SDS-PAGE and blots were probed for GRK2.

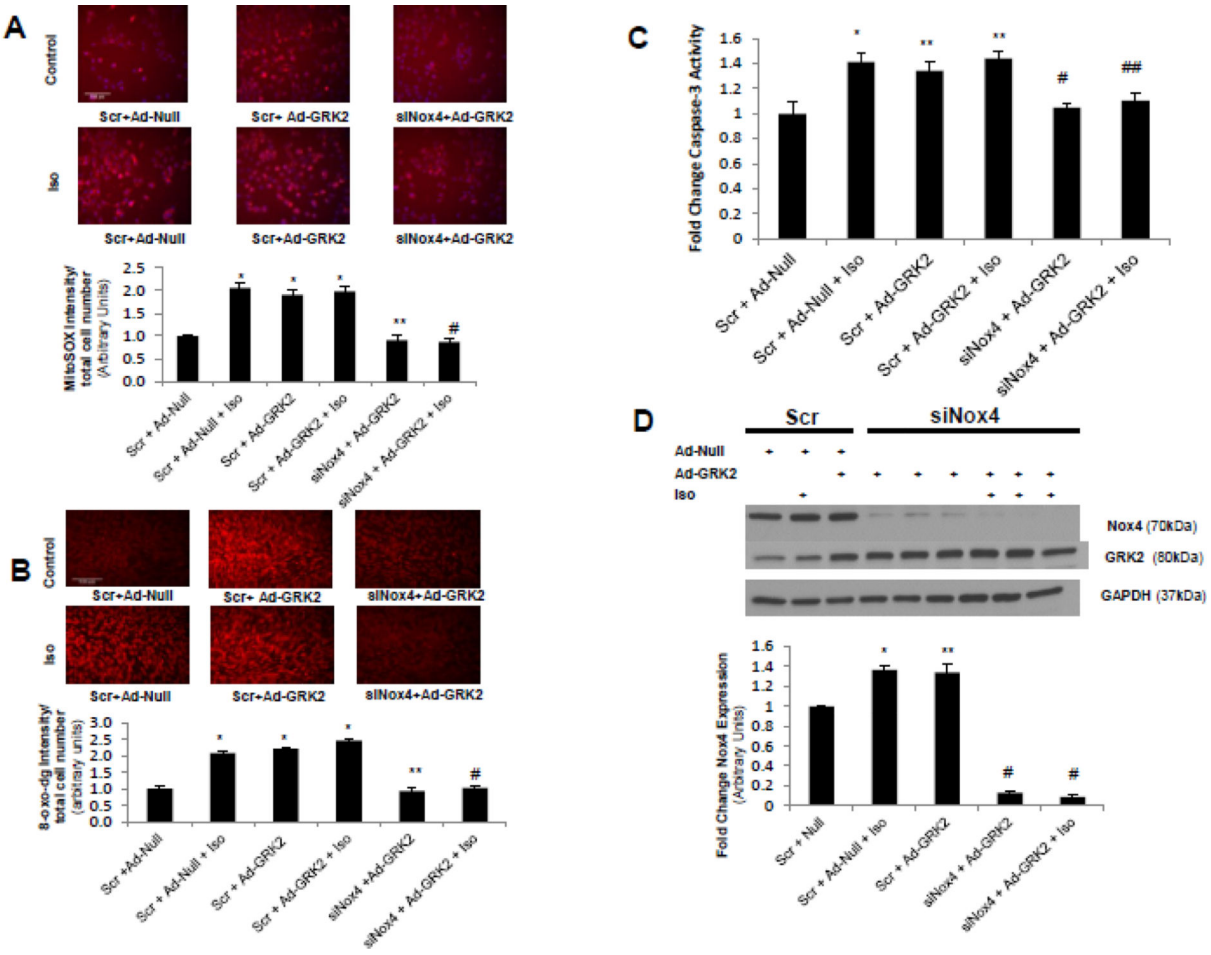


Figure 7. Oxidative stress induced by GRK2 is mediated by Nox4

A, Confocal images of H9c2 cells stained with MitoSOX (*red*) showing basal and β-AR agonist, isoproterenol (Iso) stimulated mitochondrial oxidative stress in cells infected with adenoviruses overexpressing either GRK2 (Ad-GRK2), GRK2 inhibitor, GRK2ct, (Ad-GRK2ct), or null virus (Ad-Null) and treated with siRNA to knockdown Nox4 (siNox4) or with scrambled control sequence (Scr). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n≥3; *p<0.002 vs. Scr + Ad-Null, **p=0.001 vs. Scr + Ad+GRK2, #p<0.0003 vs. Scr + Ad-GRK2 + Iso. B, Confocal images (*upper panel*) of 8-hydroxyguanine (8-oxo-dg) stained *red* Alexa Fluor 594 dye are shown basal and Iso treated H9C2 cells following transfection with both adenoviruses and siRNA. Fluorescence quantitation sown below. n=3; *p<0.001 vs. Scr + Ad-Null, **p<0.0002 vs. Scr + Ad-GRK2, #p<0.0001 vs. Scr + Ad-GRK2 + Iso. C, Caspase-3 fluorometric assay showing knockdown of Nox4 inhibits increases in caspases-3 activity in setting of GKR2 overexpression. n=3; *p=0.02 vs. Scr + Ad-Null, **p<0.04 vs. Scr + Ad-Null, #p<0.02 vs. Scr + Ad-GRK2, ##p=0.015 vs. Scr + Ad-GRK2 + Iso. *D*, Representative immunoblot (*upper panel*) showing Nox4 and GRK2 expression. GAPDH was used as a loading control. Densitometric analysis (*lower panel*) of Nox4 normalized to GAPDH. n=3; *p<0.001 vs. Scr + Ad-Null, **p=0.18 vs. Scr + Ad-Null, #p<0.0002 vs. Scr + Ad-GRK2.

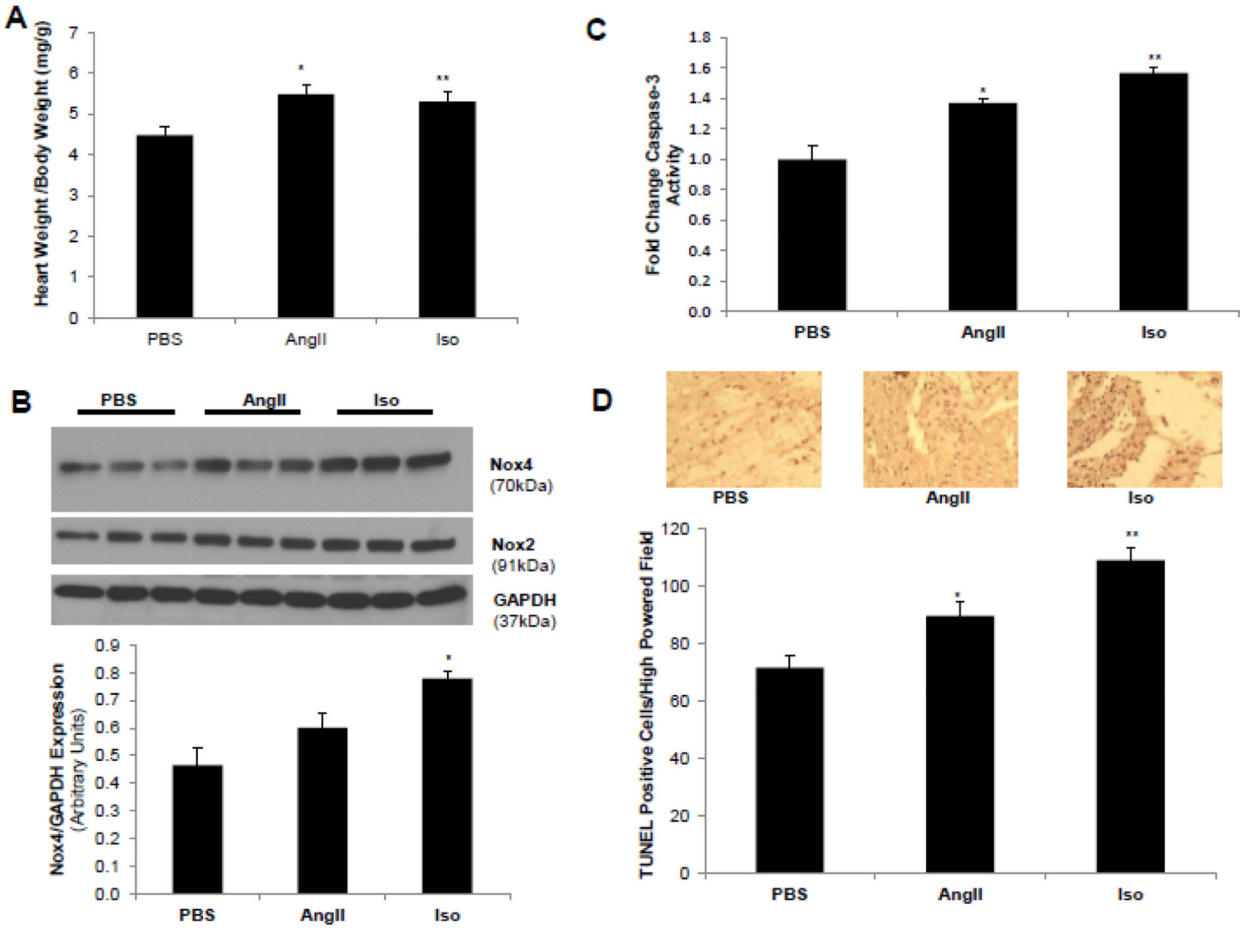


Figure 8. Chronic β-agonist treatment increases Nox4 expression and apoptosis *in vivo*

A, Heart weight:body weight of adult male wild-type C57bl6 mice following 10 days of β-agonist, isoproterenol (Iso), Angiotensin II (AngII), or PBS delivery via mini-osmotic pump. n≥6; *p<0.005 vs. PBS, **p<0.02 vs. PBS. *B*, Representative immunoblot (*upper panel*) showing myocardial Nox4 and Nox2 expression following chronic stimulation with Iso, AngII or PBS. GAPDH was used as a loading control. Densitometric analysis (*lower panel*) showing increased Nox4 expression with chronic Iso treatment. n=3; *p=0.01 vs. PBS and p=0.04 vs. AngII. AngII was not different from PBS; p=0.19. There was no significant change in Nox2 expression. C, Caspase-3 fluorometric showing increased whole heart caspase-3 activity in mice chronically treated with either AngII or Iso. n=3; *p=0.017 vs. PBS, **p<0.005 vs. PBS & p=0.013 vs. AngII. *D*, Representative photomicrographs (*upper panel*) of mice heart sections stained with TUNEL to detect apoptotic cells (*dark brown*). Quantitation of TUNEL-positive cells per high powered field shown below. n=3; *p<0.05 vs. PBS, **p<0.003 vs. PBS and p=0.04 vs. AngII.

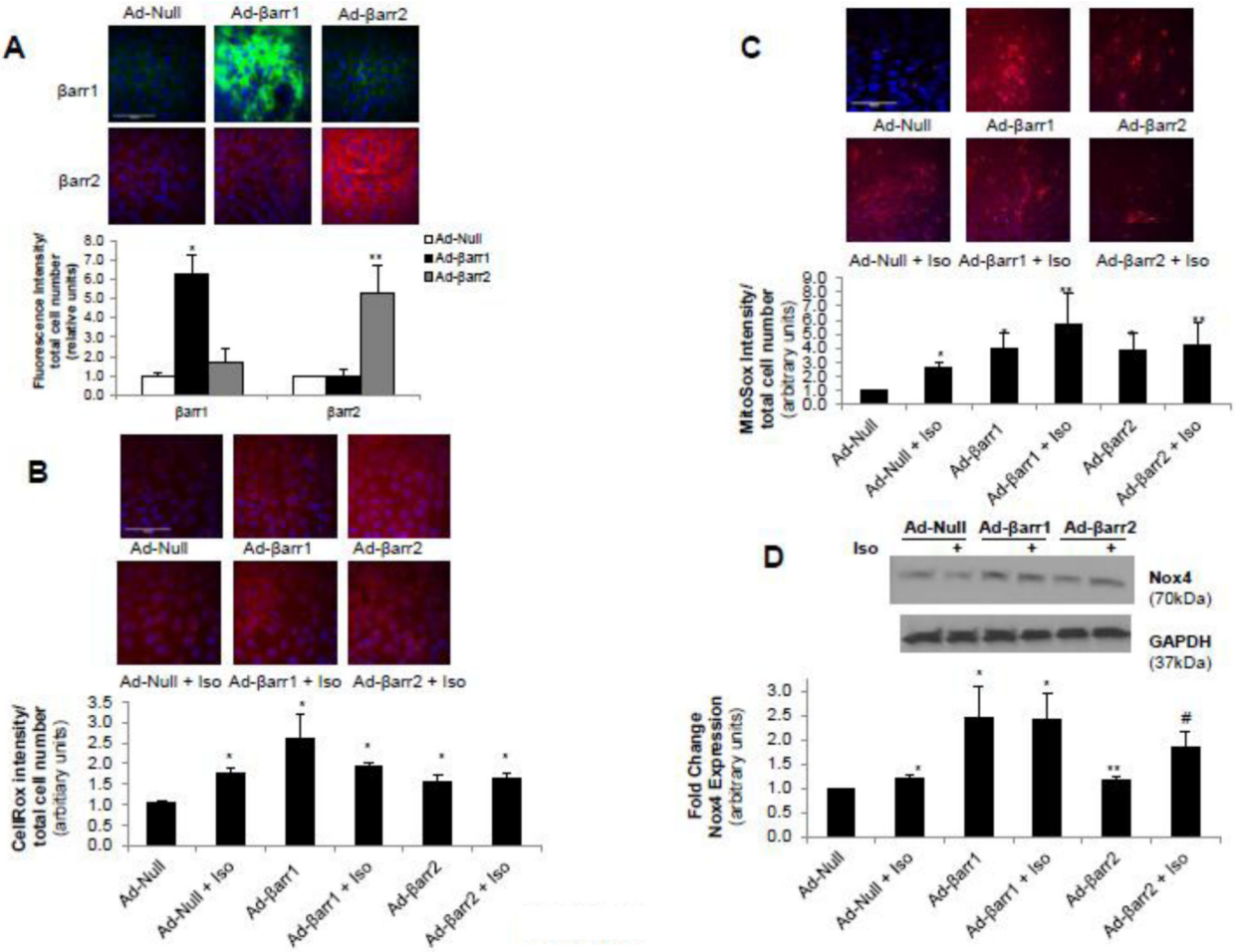


Figure 9. β-arrestin overexpression increases cellular and mitochondrial oxidative stress and upregulates Nox4

A, Confocal images (*upper panel*) of H9c2 cells following adenoviral transfection with β-arrestin1 overexpression (Ad-βarr1), β-arrestin2 overexpression (Ad-βarr2) or Null (Ad-Null) adenovirus. β-arrestin1 (βarr1) is stained with *green* with FITC, β-arrestin2 (βarr2) is stained *red* with Alexa Fluor 594 dye. Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n≥3 in each group, *p<0.01 vs. Ad-Null & vs. Ad-βarr2, **p<0.03 vs. Ad-Null & vs. Ad-βarr1. *B*, Confocal images (*upper panel*) of H9c2 cells stained with CellROX (*red*) showing basal and β-AR agonist, isoproterenol (Iso) stimulated total oxidative stress following adenoviral transfection with Ad-βarr1, Ad-βarr1, or Ad-Null. Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n≥3, *p<0.04 vs. Ad-Null. *C*, Confocal images (*upper panel*) of H9c2 cells stained with MitoSOX (*red*) showing increase in mitochondrial oxidative stress in setting of βarr1 or βarr2 overexpression. Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. n≥4, *p<0.03 vs. Ad-Null; **p<0.05 vs. Ad-Null. *D*, Representative immunoblot (*upper panel*) showing increased Nox4 expression following βarr1 or βarr2 overexpression. GAPDH was used as a loading control. Densitometric analysis shown below. n≥3; *p<0.04 vs. Ad-Null; **p<0.005 vs. Ad-Null; #p=0.013 vs. Ad-Null.

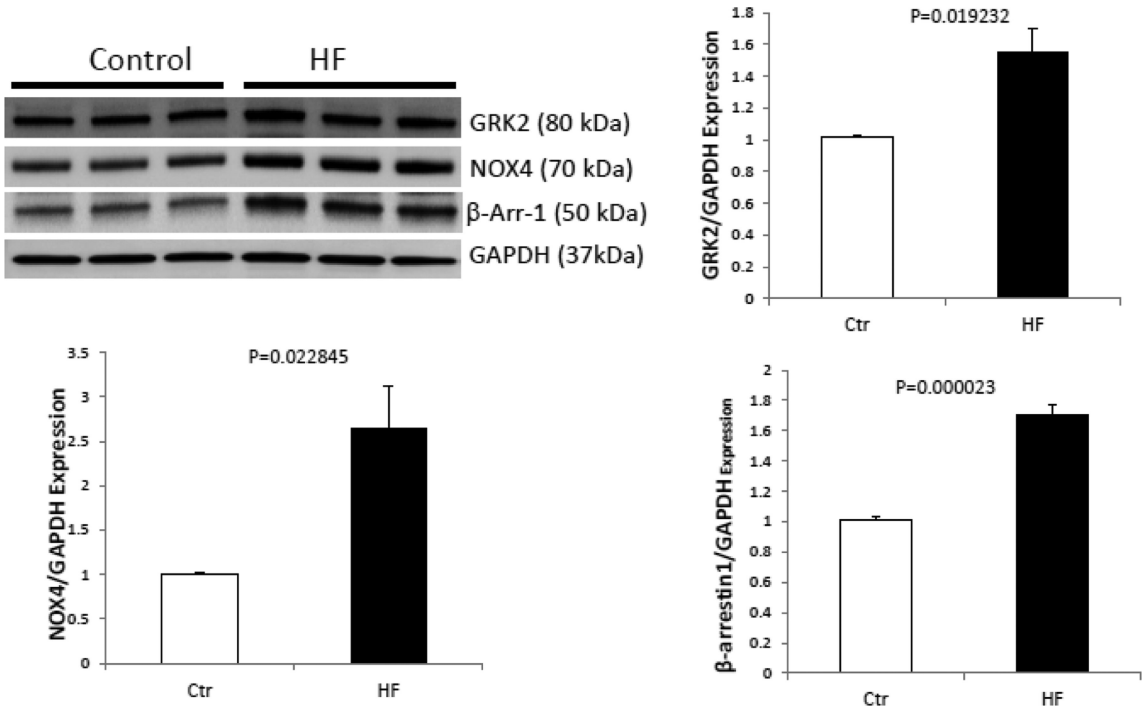


Figure 10. GRK2, NOX4 and β-arrestin1 expression is increased in HF

Representative immunoblot (*upper panel*) showing increased GRK2, NOX4 and β-arrestin1 expression in HF. GAPDH was used as a loading control. Densitometric analysis shown below. n≥3; For Nox4 p=0.02 vs. normal control, for GRK2 p=0.019 vs. normal control and for β-arrestin1 p=0.0002 vs. normal control.